A Novel Pan-Negative-Gating Modulator of KCa2/3 Channels, Fluoro-Di-Benzoate, RA-2, Inhibits Endothelium-Derived Hyperpolarization–Type Relaxation in Coronary Artery and Produces Bradycardia In Vivo

Aida Oliván-Viguera, Marta Sofía Valero, Nicole Coleman, Brandon M. Brown, Celia Laría, María Divina Murillo, José A. Gálvez, María D. Díaz-de-Villegas, Heike Wulff, Ramón Badorrey, and Ralf Köhler

Aragon Institute of Health Sciences, Zaragoza, Spain (A.O.-V., R.K.); GIMACES, Facultad de Ciencias de la Salud, Universidad San Jorge, Villanueva de Gállego, Spain (M.S.V., C.L.); Department of Pharmacology, School of Medicine, University of California Davis, Davis, California (N.C., B.M.B, H.W.); Departamento de Farmacología y Fisiología, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain (M.D.D.M.); Departamento de Catálisis y Procesos Catalíticos, Instituto de Síntesis Química y Catalización Homogénea, Consejo Superior de Investigaciones Científicas—Universidad de Zaragoza, Zaragoza, Spain (M.D.D.-V., J.A.G., R.B.); and Fundación Agencia Aragonesa para la Investigación y Desarrollo (R.K.).

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ABSTRACT

Small/intermediate conductance KCa channels (KCa2/3) are Ca2+/calmodulin regulated K+ channels that produce membrane hyperpolarization and shape neurologic, epithelial, cardiovascular, and immunologic functions. Moreover, they emerged as therapeutic targets to treat cardiovascular disease, chronic inflammation, and some cancers. Here, we aimed to generate a new pharmacophore for negative-gating modulation of KCa2/3 channels. We synthesized a series of mono- and dibenzozates and identified three dibenzozates [1,3-phenylenbis(methylene) bis(3-fluoro-4-hydroxybenzoate) (RA-2), 1,2-phenylenbis(methylene) bis(3-fluoro-4-hydroxybenzoate), and 1,4-phenylenbis(methylene) bis(3-fluoro-4-hydroxybenzoate)] with inhibitory efficacy as determined by patch clamp. Among them, RA-2 was the most drug-like and inhibited human KCa3.1 with an IC50 of 17 nM and determined by patch clamp. RA-2 at drug-like and inhibited human KCa3.1 with an IC50 of 17 nM and determined by patch clamp. Among them, RA-2 was the most drug-like and inhibited human KCa3.1 with an IC50 of 17 nM and determined by patch clamp. RA-2 had no considerable blocking effects on distantly related large-conductance KCa1.1, Kv1.2/1.3, Kv7.4, hERG, or inwardly rectifying K+ channels. In isometric myography on porcine coronary arteries, RA-2 inhibited bradykinin-induced endothelium-derived hyperpolarization (EDH)–type relaxation in U46619-precontracted rings. Blood pressure telemetry in mice showed that intraperitoneal application of RA-2 (≤100 mg/kg) did not increase blood pressure or cause gross behavioral deficits. However, RA-2 decreased heart rate by ≈145 beats per minute, which was not seen in KCa3.1−/− mice. In conclusion, we identified the KCa2/3-negative-gating modulator, RA-2, as a new pharmacophore with nanomolar potency. RA-2 may be of use to generate structurally new types of negative-gating modulators that could help to define the physiologic and pathomechanistic roles of KCa2/3 in the vasculature, central nervous system, and during inflammation in vivo.

Introduction

Small-conductance Ca2+-activated K+ channels (KCa2) (Köhler et al., 1996; Adelman et al., 2012) and the intermediate-conductance Ca2+-activated K+ channel (KCa3.1) (Ishii et al., 1997) are Ca2+/calmodulin (CaM)-regulated and voltage-independent K+ channels (Wei et al., 2005). Their activation produces solid membrane hyperpolarization that in turn influences electrical excitability and shapes calcium entry through calcium-permeable channels. KCa2 channels (subtypes 2.1, 2.2, and 2.3) are expressed in excitable tissues such as neurons, skeletal muscle, adrenal gland, and heart, and some nonexcitable tissues such as the liver and vascular endothelium, with varying subtype-specific tissue expression profiles (Wei et al., 2005). In neurons, these channels underlie the apamin-sensitive medium afterhyperpolarization and regulate firing frequency as well as learning and memory (Adelman et al., 2012). In the cardiovascular system, KCa2 channels contribute to cardiac repolarization (Li et al., 2009; Diness et al., 2010), endothelium-derived hyperpolarization (EDH)–type arterial dilation in response to increased hemodynamics (Edwards et al., 2010; Milkau et al., 2010; Wulff and Köhler, 2013), and produces solid membrane hyperpolarization that in turn influences electrical excitability and shapes calcium entry through calcium-permeable channels. KCa2 channels (subtypes 2.1, 2.2, and 2.3) are expressed in excitable tissues such as neurons, skeletal muscle, adrenal gland, and heart, and some nonexcitable tissues such as the liver and vascular endothelium, with varying subtype-specific tissue expression profiles (Wei et al., 2005). In neurons, these channels underlie the apamin-sensitive medium afterhyperpolarization and regulate firing frequency as well as learning and memory (Adelman et al., 2012). In the cardiovascular system, KCa2 channels contribute to cardiac repolarization (Li et al., 2009; Diness et al., 2010), endothelium-derived hyperpolarization (EDH)–type arterial dilation in response to increased hemodynamics (Edwards et al., 2010; Milkau et al., 2010; Wulff and Köhler, 2013), and...
provide negative feedback on sympathetic tone (Taylor et al., 2003). With respect to pathophysiological relevant functions in humans, recent evidence suggests a role, particularly of KCa3.1, in lone atrial fibrillation (Diness et al., 2010; Ellinor et al., 2010), cancer cell migration and metastasis (Chantôme et al., 2013), and overactive bladder (Soder et al., 2013).

KCa3.1 channels are mostly expressed in nonexcitable tissues such as red and white blood cell lineages, secretory epithelia, and the vascular endothelium (Devor et al., 1996; Köhler et al., 2000; Wei et al., 2005; Wulff and Köhler, 2013). Here, hyperpolarization and K+ efflux through Ca2+-activation KCa3.1 channels regulate cell volume regulation (Vandorpe et al., 1998), fluid secretion (Devor et al., 1996), and—together with KCa2.3 channels—EDH-type arterial dilation, specifically to acetylcholine stimulation (Edwards et al., 2010; Wulff and Köhler, 2013). Initially, KCa3.1 was believed to be an exclusively nonneuronal channel (Ishii et al., 1997; Wei et al., 2005). However, recent evidence suggests possible expression in cerebellar Purkinje cells in rats (Engbers et al., 2012) and a role of the channel in behavior as suggested by the locomotor hyperactivity in KCa3.1−/− mice (Lambertsen et al., 2012). KCa3.1 channels have been patho-mechanistically implicated in human disease such as arterial endothelial dysfunction (Féletou et al., 2010); cancer growth; cancer cell migration, metastasis (D’Alessandro et al., 2013), and neo-angiogenesis (Grigc et al., 2005); organ fibrosis (Grigc et al., 2009); atherosclerosis (Toyama et al., 2008); neointima formation (Köhler et al., 2003; Tharp et al., 2005); organ fibrosis (Köhler et al., 2010; D’Alessandro et al., 2013), and positive-gating modulators (activators) of KCa3.1, such as naphtho[2,1-d]thiazol-2-ylamine (SKA-31) (Sankaranarayanan et al., 2009) and 5-methylnaphtho[2,1-c]pyrazole (TRAM-34) (Wulff et al., 2001), 4-(tert-butyl-phenoxyethyl)-[1,2,4]triazolo[1,5-a]pyrimidin-2-yl-N₂-methoxyformamide (−)-B-TPMF (Hougaard et al., 2012) have been described for KCa2.3 channels. Recently, our screening of a series of phenols and polyphenols identified the synthetic fluoro-tri-benzoic ester, 13b (Lamoral-Theys et al., 2010) (Fig. 1A), as a negative-gating modulator of KCa2.3 channels (Oliván-Viguera et al., 2013). However, a disadvantage of 13b is that its high molecular weight (582) and its log P value of 6.0 violate the Lipinski et al. (2001) rule of five and make it unlikely that the compound will have oral bioavailability. Moreover, the structure-activity relationship accounting for channel inhibition has not been characterized yet. Therefore, we decided to explore the structure-activity relationship by synthesizing less lipophilic and smaller analogs. Three compounds (1,3-phenylenes(methylene) bis(3-fluoro-4-hydroxybenzoate) (RA-2), 1,2-phenylenes(methylene) bis(3-fluoro-4-hydroxybenzoate) (RA-3), and 1,4-phenylenes(methylene) bis(3-fluoro-4-hydroxybenzoate) (RA-4)) (Fig. 1B) were identified as KCa2,3 pan inhibitors with negative-gating modulator properties and potencies in the nanomolar range. RA-2 inhibited EDH-type dilations in porcine coronary arteries (PCAs) and was found not to increase blood pressure in telemetry recordings despite reducing heart rate (HR).

Materials and Methods

Synthesis of Mono- and Dibenzoates. As shown in Fig. 1B, monobenzoate benzyl 3-fluoro-4-hydroxybenzoate (RA-1) was obtained by reaction of benzyl bromide with 3-fluoro-4-hydroxybenzoic acid. Dibenzoates RA-2, RA-3, and RA-4 were obtained by reaction of the corresponding

ABBREVIATIONS: BK, bradykinin; bs, broad singlet; CaM, calmodulin; d, doublet; dd, doublet of doublets; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDH, endothelium-derived hyperpolarization; ESI, electrospray ionization; HEK, human embryonic kidney; HR, heart rate; HRMS, high-resolution mass spectra; IR, infrared; KCa2, Ca2+-activated K+; KCa3.1, small-conductance Ca2+-activated K+; KCa2,3, intermediate-conductance Ca2+-activated K+; m, multiplet; PCA, porcine coronary artery; PCAEC, porcine coronary artery endothelial cell; s, singlet; RA-1, benzyl 3-fluoro-4-hydroxybenzoate; RA-2, 1,3-phenylenes(methylene) bis(3-fluoro-4-hydroxybenzoate); RA-3, 1,2-phenylenes(methylene) bis(3-fluoro-4-hydroxybenzoate); RA-4, 1,4-phenylenes(methylene) bis(3-fluoro-4-hydroxybenzoate); RA-5, 1,3-phenylenes(methylene) bis(4-acetamido-3-fluoro-benzoate); RA-6, 5-(hydroxymethyl)-1,3-phenylenes(methylene) bis(3-fluoro-4-hydroxybenzoate); SKA-31, naphtho[1,2-d]thiazol-2-ylamine; TRAM-34, 1-[2-chlorophenyl] (diphenyl)methyl]-1H-pyrazole; U46619, (Z)-7-[[1S,4R,5R,6S]-5-[[(E,3S)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]-hept-5-enolic acid; WT, wild-type.
benzoates. (A) Structure of the parent compound, 13b. (B) Scheme of the syntheses of the corresponding mono- or dibenzoates in 33.

![Fig. 1](http://example.com/fig1.png)

Fig. 1. Synthesis, structures, and selected properties of mono- and difluoro benzoates. (A) Structure of the parent compound, 13b. (B) Scheme of the syntheses of mono- and difluoro benzoates. RA-1 to RA-6, together with molecular weights and log P values.

**General Procedures and Physical Data.** Whenever possible, the reactions were monitored by thin layer chromatography, which was performed on precoated silica gel polyester plates. The products were visualized using UV light (254 nm) or ethanolic phosphomolybdic acid solution followed by heating. Column chromatography was performed using silica gel (Kieselgel 60, 230–400 mesh; Sigma-Aldrich). Melting points were determined in open capillaries using a Gallenkamp capillary melting point apparatus and were not corrected. Fourier transform infrared (IR) spectra were recorded as KBr pellets using a Thermo Nicolet Avatar 360 Fourier transform IR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA); νmax values expressed in cm⁻¹ are given for the main absorption bands. ¹H NMR and ¹³C NMR spectra were acquired on a Bruker AV-400 (Wageningen UR, The Netherlands) spectrometer operating at 400 MHz for ¹H NMR, 100 MHz for ¹³C NMR, and 376 MHz for ¹⁹F NMR at room temperature using a 5 mm probe. The chemical shifts (δ) are reported in parts per million and were referenced to the residual solvent peak. Coupling constants (J) are quoted in Hertz. The following abbreviations are used: s, singlet; d, doublet; m, multiplet; bs, broad singlet; and dd, doublet of doublets. High-resolution mass spectra (HRMS) were recorded using a Bruker Daltonics MicroToF-Q instrument from methanolic solutions unless otherwise indicated using the positive electrospray ionization (ESI⁺) mode.

**Synthesis of 4-Acetamido-3-Fluorobenzoic Acid.** A mixture of 4-amino-3-fluorobenzoic acid (233 mg, 1.5 mmol) and acetic anhydride (459 mg, 425 μl, 4.5 mmol) in anhydrous pyridine (7 ml) was heated at 60°C overnight. Then, the mixture was concentrated in vacuo. Over the residue, water (6 ml) was added and then the aqueous solution was acidified with HCl 2N until pH = 1. The product precipitated and was collected by filtration and dried providing 281 mg (95%) of compound 4-acetamido-3-fluorobenzoic acid as a slightly brownish solid. ¹H NMR (CD3OD, 400 MHz) δ 2.20 (s, 3H), 7.74 (dd, 1H, J = 8.1, 1.8), 8.20 (dd, 1H, J = 8.1, J = 8.1); ¹³C NMR (CD3OD, 100 MHz) δ 24.2, 117.3 (d, 21.1), 123.6, 127.1 (d, 3.1), 128.2, 132.2 (d, 11.3), 153.8 (d, 243.9), 167.8 (d, 2.6), 171.5; ¹⁹F NMR (CD3OD, 376 MHz) δ -128.6.

**Synthesis of RA-1.** A mixture of benzyl bromide (171 mg, 1 mmol), 3-fluoro-4-hydroxybenzoic acid (156 mg, 1.0 mmol), and NaHCO₃ (sodium 101 mg, 1.2 mmol) in anhydrous DMF (15 ml) under an argon atmosphere was heated at 105°C under argon atmosphere overnight. The mixture was cooled and then saturated aqueous NaHCO₃ (7 ml), saturated aqueous NaCl (10 ml), and AcOEt (50 ml) were added. The mixture was filtered through a pad of Celite and after decantation the aqueous layer was extracted with AcOEt (50 ml). The combined organic layers were dried over anhydrous MgSO₄ and concentrated in vacuo. Finally, the crude product was purified by silica gel column chromatography (eluents: AcOEt/hexane, 1:3) to afford 147 mg (60%) of compound RA-1 as a white solid. M.p. 93°C.

**Synthesis of Bis(3-fluoro-4-hydroxybenzoates) from Dibromomethylenbenzene Derivatives.** A mixture of the corresponding dibromide derivate (1.0 mmol), 1,3-bis(bromomethyl)benzene with 3-fluoro-4-hydroxybenzoic acid (343 mg, 2.2 mmol), and NaHCO₃ (210 mg, 2.5 mmol) in anhydrous DMF (15 ml) under an argon atmosphere was heated at 105°C overnight. The mixture was cooled and then saturated aqueous NaHCO₃ (7 ml), saturated aqueous NaCl (10 ml), and AcOEt (50 ml) were added. The mixture was filtered through a pad of Celite and after decantation the aqueous layer was extracted with AcOEt (2 × 20 ml). The combined organic layers were dried over anhydrous MgSO₄ and concentrated in vacuo. Finally, the crude product was purified by silica gel column chromatography (eluents: AcOEt/hexane, 1:3).
s (4H), 6.85–6.95 (m, 2H), 7.34–7.35 (m, 3H), 7.46 (bs, 1H), 7.60–7.70 (m, 4H); 13C NMR (CD3OD, 100 MHz) δ 67.3, 118.3, (d, J = 20.0), 118.0, (d, J = 3.0), 122.5 (d, J = 6.0), 127.9 (d, J = 3.0), 132.0, 131.4, 136.3, 151.5 (d, J = 12.9), 152.2 (d, J = 240.3), 166.8 (d, J = 2.7); 19F NMR (CD3OD, 376 MHz) δ -138.5. HRMS (ESI+) calculated for C26H24F2NaO9 [M + Na]+ 519.1338; found 519.1307.

Synthesis of RA-5. A mixture of 1,3-bis(bromomethyl)benzene (132 mg, 0.5 mmol), 4-acetamido-3-fluorobenzoic acid (217 mg, 1.1 mmol) and K2CO3 (173 mg, 1.25 mmol) in anhydrous DMF (7 ml) under an argon atmosphere was heated at 105°C overnight. The mixture was cooled and then saturated aqueous NaHCO3 (3 ml), saturated aqueous NaCl (5 ml), and AcOEt (25 ml) were added. The obtained mixture was filtered through a pad of Celite and after decantation the aqueous layer was extracted with AcOEt (2 × 10 ml). The combined organic layers were dried over anhydrous MgSO4 and concentrated in vacuo. Recrystallization from methanol afforded 125 mg (50%) of the compound RA-5 as a brownish solid. M. p. 190–191°C; IR (KBr): νmax = 3303, 3292, 3192, 1719, 1676, 1620, 1607, 1541; 1H NMR (CD3OD, 400 MHz) δ 5.27 (d, J = 12.8), 7.30 (d, J = 6.0), 7.29 (d, J = 12.9), 7.24, 7.57, 11.83 (d, J = 20.0), 118.5 (d, J = 3.0), 122.7 (J = 6.0), 127.4, 127.5, 128.0 (d, J = 3.0), 138.3, 143.8, 151.4 (d, J = 12.8), 152.3 (d, J = 240.3), 166.9 (d, J = 2.6); 13C NMR (CD3OD, 376 MHz) δ -138.5; HRMS (ESI+) calculated for C22H16F2NaO6 [M + Na]+ 437.0807; found 437.0809.

Cell Lines. Human embryonic kidney cells (HEK293) stably expressing hKCa3.1 (a kind gift from Dr. Khaled M. Houamed, University of Chicago) (Cao and Houamed, 1999); HEK293.B2 (Herkenham et al., 1995) were seeded on cover slips in NaCl bath solution (see below), and used for electrophysiological measurements within the same day.

Compounds and Chemicals. Compounds for synthesis and experimentation were purchased from Sigma-Aldrich, Tocris (Bristol, United Kingdom), Fluorochem, or Alfa Aesar. TRAM-34 (Wulff et al., 2000) and SKA-31 (Sankaranarayanan et al., 2009) were synthesized in the Wulff laboratory (Pharmacology, University of California at Davis, CA). Stock solutions (at 1 or 10 mM) of all compounds were prepared with dimethylsulfoxide (DMSO). The final DMSO concentration did not exceed 0.5% in single experiments testing one or more compounds.

Patch-Clamp Electrophysiology. Inside-out and whole-cell membrane currents were recorded using an EPC10-USB patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany), U-ramps (~100 to 100 mV, 1 second), and Patchmaster software (HEKA Electronics) as described in more detail previously (Oliván-Vigueria et al., 2013). The amplitudes of K+-outward currents were measured at 0 mV.

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Myography on PCAs. Isometric myography on PCA rings was done as described in detail previously (Alda et al., 2009; Valero et al., 2011). In brief, rings of arteries were mounted onto an isometric force transducer (Pioden UFI, Graham Bell House, Canterbury, UK). The bath containing Krebs buffer (37°C; equilibrated with 95% O2/5% CO2) consisted of (in mM): NaCl 120, NaHCO3 24.5, CaCl2 2.4, KCl 4.7, MgSO4 1.7, and 10 HEPES (adjusted to pH 7.4). The extracellular solutions contained (in mM): 154 KCl, 10 HEPES (pH = 7.4), 2 CaCl2, 1 MgCl2, Solutions on the intracellular side contained (in mM): 154 KCl, 10 HEPES (pH = 7.2), 10 EGTA, 1.75 MgSO4, 1 CaCl2, 10 glucose, and 10 HEPES (to adjust pH to 7.4 with NaOH). For calculation of the IC50 values, data points were fitted using the dose-response equation: y = A2 + (A1 – A2)/(1 + ([x]/Kd)) or the Boltzmann equation: y = A2 + (A1 – A2)/(1 + [exp(-x/a0/dx)]. The inside-out experiments on hKCa3.1 shown in Fig. 2D were performed in symmetrical K+.

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Hearts were kindly provided by the local abattoir (Mercarazagoza, Zaragoza). Prior to patch-clamp experimentation, cells were trypsinized, seeded on cover slips in a NaCl bath solution (see below), and used for electrophysiological measurements within the same day.
Blood Pressure Telemetry. Telemetry was performed as described previously (Brähler et al., 2009; Radtke et al., 2013). Animal protocols were in accordance with Animal Research: Reporting of In Vivo Experiments guidelines and approved by the Institutional Animal Care and Use Committee of the University of Zaragoza and IACS (Comisión Ética Asesora [CEA]; permit no. PI01/13). In brief, TA11PA-C10 pressure transducers (Data Sciences International, St. Paul, MN) were implanted into the left carotid artery of four adult female wild-type (WT) and three adult female K_Ca3.1/2 (Brähler et al., 2009) under deep anesthesia as described previously (Radtke et al., 2013). Mice were allowed to recover for 10 days until reaching normal day night rhythm. Mice had free access to tap water and standard chow. Telemetry data were recorded over 1 minute every 10 minutes over 24 hours and averaged. Data were analyzed using the Data Sciences International software.

The compound or vehicle (peanut oil) was injected during the third hour of the dark phase (activity phase) and we collected telemetry data after 20–30 minutes after injection. To minimize stress and pain caused by intraperitoneal injections, mice were briefly anesthetized by isoflurane inhalation. After a first injection, animals were reused for injections of a higher dose of RA-2 or vehicle. Preparation and injection of RA-2: Appropriate amounts of RA-2 were dissolved in warmed peanut oil (Sigma-Aldrich) to give a dose of 3, 30, or 100 mg/kg. Injection volume was ≤600 μl.

Pharmacokinetics. RA-2 was dissolved in peanut oil and 30 mg/kg were administered intraperitoneally to female C57Bl6J mice (20–30 g, 3–5 month old, n = 3 per time point) as described above. Mice were sacrificed at 1, 2, 4, 8, 24, or 48 hours after injection. Blood was taken by puncture of the right ventricle under CO_2 anesthesia (permit no. PI01/13), transferred into EDTA-containing tubes, and stored on ice until further processing. EDTA-blood was centrifuged at 1000 g for 10 minutes and 4°C and plasma was stored at −20°C. Tissue samples (liver, brain, and femoral skeletal muscle) were excised, frozen, and stored at −20°C until use.

Plasma (50 μl) was added to 950 μl acetonitrile, vortexed and centrifuged at 13,500g for 30 minutes at room temperature to precipitate the protein. The supernatant was transferred to a 4 ml vial, concentrated to dryness and reconstituted with acetonitrile to 150 μl. Tissue samples (200 mg) were homogenized in 1 ml of H_2O with a Brinkman Kinematica PT 1600E homogenizer (Brinkman Kinematica, Eschbach, Germany) and protein precipitated with 1 ml of acetonitrile. The samples were then centrifuged at 13,500g for...
30 minutes. The supernatants were concentrated to dryness and reconstituted in 200 μl of acetonitrile. Liquid chromatography and mass spectrometry analysis was performed with a Waters Acquity UPLC (Waters, New York) equipped with a Acquity UPLC BEH 1.7 μm RP-8, 2.1 × 150 mm column (Waters) interfaced to a TSQ Quantum Access Max mass spectrometer (ThermoFisher Scientific, Waltham, MA). Using an atmospheric pressure chemical ionization mass spectrometer and selective reaction monitoring (capillary temperature 327°C; vaporization temperature 530°C; collision energy 35 eV, negative ion mode), RA-2 was quantified by its base peak of 110.2 m/z and its concentration was calculated with a four-point calibration curve from 10 nM to 1 μM. The mobile phase consisted of acetonitrile and water, both containing 0.2% acetic acid with a flow rate of 0.300 ml/min. The gradient was ramped from 95/5 water/acetonitrile to 5/95 acetonitrile/water over 2 minutes and returned to 95/5 water/acetonitrile after 6 minutes. RA-2 was introduced onto the column with an injection volume of 6 μl. Using these conditions, RA-2 had a retention time of 3.78 minutes. Liver metabolites were analyzed under full scan mode without collision energy with a mass range of 150–1500 m/z. The mobile phase consisted of an isocratic gradient (90/10 acetonitrile/water). Under these conditions, RA-2 had a retention time of 1.76 minutes and liver metabolites eluted at 1.74, 2.71, and 3.11 minutes.

**Statistics.** Data are given as mean ± S.E.M. if not stated otherwise. For comparison of data sets we used the unpaired Student’s t test or one-way analysis of variance followed by the Tukey post hoc test in the case of multiple comparisons. P values of <0.05 were considered significant.

**Results**

We previously identified 13b (Fig. 1A) as a negative-gating modulator of KCa2/3 channels (Oliván-Viguera et al., 2013). However, 13b’s drug likeness is poor because of its high molecular weight and high lipophilicity, limiting its in vivo bioavailability. Therefore, we synthesized a series of smaller and less lipophilic mono- and dibenzoates that met Lipinski’s rule of five for a drug-like compound (Lipinski et al., 2001) (Fig. 1). RA-1, RA-2, RA-3, RA-4, and RA-6 (Fig. 1B) were obtained from the corresponding mono- or dibromide-3-fluoro-4-hydroxybenzoic acid. RA-5 was obtained from 1,3-bis(bromomethyl)benzene and 4-acetamido-3-fluorobenzoic acid, which in turn was obtained by acetylation of 4-amino-3-fluorobenzoic acid. RA-1, RA-2, RA-3, RA-4, and RA-6 (Fig. 1B) were obtained from the corresponding mono- or dibromide-3-fluoro-4-hydroxybenzoic acid. RA-5 was obtained from 1,3-bis(bromomethyl)benzene and 4-acetamido-3-fluorobenzoic acid, which in turn was obtained by acetylation of 4-amino-3-fluorobenzoic acid.

**Electrophysiology.** We performed inside-out and whole-cell patch-clamp experiments on cloned hKCa3,1 and hKCa2,3 as well as native KCa3,1/KCa2,3 in PCAECs and a series of distantly related K channels to test efficacy and selectivity. Removal of lipophilic branches from the parent compound led to the following results: Compound RA-1 with only one benzoate ring had no considerable inhibitory activity on KCa3,1 (Table 1). The 1,3-dibenzoate RA-2 was a potent KCa3,1 and KCa2,3 inhibitor with IC50 values of 17 ± 3 nM and 2 ± 1 nM as determined in inside-out recordings on hKCa3,1 in HEK-293 cells and hKCa2,3 in COS7 cells (Fig. 2, A and B; for numeric data, see Supplemental Table 1 and Table 1). RA-2 similarly inhibited cloned hKCa2,1 and rKCa2,2 at nanomolar concentrations (Supplemental Table 1). We obtained similar IC50 values in whole-cell experiments on murine KCa3,1 expressed in 3T3-fibroblasts (Supplemental Fig. 1A). The 1,2- and 1,4-dibenzoate derivatives, RA-3 and RA-4, showed a similar potency with an IC50 value for RA-3 of 6 ± 2 nM (Supplemental Fig. 1B; Table 1). Compound RA-5, in which the hydroxyl group on the benzoic acid moiety had been substituted by an acetamido group (Fig. 1B), had no considerable inhibitory activity (Table 1). Introduction of a hydroxymethyl substituent in the 5-position of the central aromatic core (RA-6, Fig. 1B) led to a loss of activity (Table 1). It is noteworthy that 3-fluorobenzoic moieties (4-hydroxy-3-fluorobenzoic acid and 4-amino-3-fluorobenzoic acid) and 1,3-phenylenedimethanol as potential metabolites of RA-2/RA-5 hydrolysis, respectively, did not modify KCa3,1 currents.

Similar to our previous observations with the template 13b (Oliván-Viguera et al., 2013), we found that the positive-gating modulator, SKA-31, was capable of reversing the inhibition caused by nanomolar but not micromolar concentrations of RA-2 (Fig. 2C). This suggested (1) antagonism of the two compounds; (2) inhibitory actions of RA-2 similar to that of 13b; and (3) that RA-2 acted as a negative-gating modulator again similar to 13b (Oliván-Viguera et al., 2013). To determine if the effects of RA-2 were Ca2+ dependent as was the case with previously described negative-gating modulators such as NS8593 and its derivatives (Jenkins et al., 2011), we performed inside-out experiments, in which we varied the intracellular [Ca2+]i concentration and investigated the ability of 100 nM of RA-2 to inhibit KCa3,1 currents. As shown in Fig. 2D, 100 nM of RA-2 nearly completely inhibited the current elicited by 500 nM [Ca2+]i, while the same concentration had a smaller effect in the presence of saturating 30 μM [Ca2+]i, Ca2+ concentration-response curves obtained in the presence and absence of 100 nM of RA-2 (Fig. 2D, right-hand side) revealed that RA-2 shifted the curve to the right but also reduced the maximal current at 30 μM, suggesting that the compound exerted negative-gating modulation of KCa3,1.

With respect to selectivity, we found that RA-2 had no considerable inhibitory or activating effects at a concentration of 1 μM on the distantly related human KCa1,1 channel in

<table>
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<tr>
<th><strong>TABLE 1</strong></th>
<th><strong>Inhibitory efficacy of mono- and difluorobenzoates on hKCa3,1</strong></th>
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<tbody>
<tr>
<td><strong>Compound</strong></td>
<td><strong>IC50</strong></td>
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<tr>
<td>4-Amino-3-fluorobenzoic acid</td>
<td>NT</td>
</tr>
<tr>
<td>4-Hydroxy-3-fluorobenzoic acid</td>
<td>NT</td>
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<tr>
<td>1,3-Phenylenedimethanol</td>
<td>NT</td>
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<tr>
<td>RA-1</td>
<td>94 ± 1</td>
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<tr>
<td>RA-2</td>
<td>17 ± 3 nM</td>
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<tr>
<td>RA-3</td>
<td>6 ± 2 nM</td>
</tr>
<tr>
<td>RA-4</td>
<td>15 ± 6</td>
</tr>
<tr>
<td>RA-5</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>RA-6</td>
<td>83 ± 3</td>
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NT, not tested.
U251 glioblastoma cells; cloned Kv1.2, Kv1.3, and Kv7.4 channels; the important cardiac hERG channels (Kv11.1); or native inwardly rectifying K⁺ channels in U251 cells. The data are summarized in Supplemental Table 1 and representative recordings are shown in Supplemental Figure 2.

**Patch Clamp on Porcine Coronary Endothelium and Isometric Myography.** Considering RA-2 as one of the drug-like compounds of this series, we continued evaluating functional activity in an ex vivo test system, i.e., PCAs (Oliván-Viguera et al., 2013), in which KCa2/3 channels have been suggested to initiate—at least in part (Ge et al., 2000)—the so-called EDH-type of endothelium-dependent vasorelaxation (Edwards et al., 2010). In the present study, we first measured endogenous KCa2 and KCa3.1 currents in freshly isolated PCAEC and we found that 1 μM RA-2 virtually abolished SKA-31–activated composite KCa2/KCa3.1 currents and also fully inhibited the TRAM-34–insensitive KCa2-mediated current in these cells (Fig. 3A).

Our isometric myography experiments in the presence of blockers of NO and prostacyclin synthesis (to specifically study EDH-type relaxation) showed that RA-2 at 1 μM did not modulate basal tone in these arteries (data not shown). However, RA-2 almost abolished the BK-induced relaxation in rings being strongly precontracted with a vasospasmic agent, the thromboxane analog, U46619 (Fig. 3B). RA-2 did not modulate sodium nitroprusside–induced and endothelium-independent relaxation of 60 mM KCl-contracted rings (data not shown). Taken together, RA-2 showed activity in PCAs by inhibiting EDH-type endothelium-dependent relaxation.

**Systemic Cardiovascular Effects of RA-2.** In keeping with the expression of KCa3.1 and KCa2.3 channels in the vascular endothelium and their proposed roles in systemic cardiovascular regulation (Brähler et al., 2009), we next evaluated cardiovascular activity and selectivity of the pan-KCa2/3–negative-gating modulator, RA-2, by blood pressure telemetry in WT and KCa3.1/−/− mice (Brähler et al., 2009). If compared with the vehicle, intraperitoneal injections of 3, 30, or 100 mg/kg RA-2 did not significantly change mean arterial blood pressure over 24 hours in the WT mice (Fig. 4A, for 30 mg/kg, and Supplemental Fig. 3A, for 3 and 100 mg/kg). However, RA-2 at 30 and 100 mg/kg significantly reduced HR. The reduction in HR started apparently 30–60 minutes after injection and reached lowest levels (~Δ145 beats per minute) at the end of the activity phase and during the resting phase (Fig. 4B, Supplemental Fig. 3A, lower-right panel). RA-2 at the lower dose of 3 mg/kg produced a smaller reduction in HR (Supplemental Fig. 3A, lower-left panel). Besides the lower HR, wave forms were similar in the presence or absence of RA-2 and are shown in Supplemental Fig. 3B.

We next evaluated to which extent the HR reducing effects of RA-2 in the WT mice depended on KCa3.1 channels and performed telemetry in KCa3.1/−/− mice. Similar to WT mice, RA-2 at 30 mg/kg did not change the mean arterial blood pressure in the KCa3.1−/− mice (Fig. 4C, left-hand side). However, we did not find a reduction in HR in KCa3.1−/− mice (P < 0.05; Fig. 4D, right-hand side). Together, the telemetry monitoring revealed that RA-2 had no gross deleterious effects on blood pressure. Nonetheless, it is noteworthy that RA-2 reduced HR in a KCa3.1-dependent manner.

**Pharmacokinetics of RA-2.** Analysis of tissue concentrations and distribution of RA-2 in plasma, brain, skeletal muscle, fat, and liver by a combination of ultra-high performance liquid
chromatography and mass spectrometry (Fig. 5A) revealed that RA-2 plasma concentration was 136 nM at 1 hour after injection and 18 nM at 2 hours after injection, and that the compound was not detectable at later time points (Fig. 5B). RA-2 went rapidly into tissue (skeletal muscle, brain, and fat), where at 1 hour it reached concentrations of 1.7 μM (skeletal muscle), 91 nM (brain), and 6 μM (fat) (Fig. 5, C and D). As expected for an ester, RA-2 was rapidly cleared from the circulating blood within 1 hour (Fig. 5B), presumably via hydrolysis by plasma and hepatic esterases as indicated by the absence of RA-2 in liver and high amounts of possible metabolites in this tissue (Fig. 6). Compounds with the masses shown in Fig. 6 were identified in liver homogenate and were present in estimated concentrations of 1 to approximately 50 μM but could not be precisely quantified without reference compounds. The metabolites were likely eliminated via the bile since metabolites were not detectable in plasma at the same time point. In conclusion, intraperitoneal administration of RA-2 yielded tissue concentrations above the plasma at the same time point. In conclusion, intraperitoneal administration of RA-2 yielded tissue concentrations above the plasma at the same time point.

**Discussion**

The purpose of the present study was to synthesize potent and structurally novel pharmacophores for negative-gating modulation of KCa2/3 channels. We succeeded in identifying three fluorodibenzoates that inhibited KCa2/3 channels in the low nanomolar range and in a Ca2+-concentration-dependent manner and exhibited antagonism with a positive-gating modulator. The most drug-like compound RA-2 inhibited KCa2/3-initiated EDH-type relaxation and in vivo treatments did not show acute toxicity, although we found a KCa2/3-dependent reduction of HR. Compared with 13b (Lamoral-Theys et al., 2010) that served as a template for structural modifications, compounds RA-2, RA-3, and RA-4, in which two of the three 3-fluoro-4-hydroxybenzoxoyloxymethyl substituents in 13b were conserved, showed inhibitory activity. RA-2 with both substituents in the 1,3 position was equally potent in inhibiting KCa3.1 (RA-2: 17 nM versus 13b: 19 nM) (Oliván-Viguera et al., 2013). In line with the notion that RA-2 is a pan-negative KCa2/3 modulator, all three subtypes of KCa2/3 channels were inhibited by RA-2 at nanomolar concentrations, although the IC50 value was higher for RA-2 (2 nM) than for 13b (360 pM) in the case of hKCa2.3. The structurally very similar compounds RA-3 and RA-4 with both substituents in the 1,2 and 1,4- positions, respectively, were also found to be potent KCa3.1 inhibitors. A major change in the structure such as the absence of additional 3-fluoro-4-hydroxybenzoxoyloxymethyl substituent (as in RA-1), the substitution of the hydroxyl groups by acetamido groups on the benzoic acid moieties (as in RA-5), or the introduction of a hydroxymethyl substituent in the 5-position of the central aromatic core (as in RA-6) gave lower log P values (4.7) similar to RA-2.

Together, the three structurally similar compounds, RA-2, RA-3, and RA-4 were identified as potential pharmacophores for pan-negative modulation of KCa2/3 channels that had a better chemical profile with a lower molecular weight of 414 Da (RA-2, RA-3, and RA-4) and lower log P values of 4.7 than the starting compound 13b (mol. wt. = 582; log P = 6.0), suggesting in vivo utility.

Similar to 13b, RA-2 acted as a negative-gating modulator as concluded from the substantially more potent inhibition at nanomolar Ca2+ and the antagonism (relief of channel inhibition) by the positive-gating modulator, SKA-31. Structurally, the negative-gating modulation by RA-2 and its
antagonism by the positive-gating modulator, SKA-31, could occur at the interface of CaM and the cytosolic C-terminal CaM-binding domain of the channels. Indeed, docking experiments using co-crystals of CaM and the C-terminal CaM-binding domain of KCa2.2 (Zhang et al., 2012) revealed that NS309 and 1-EBIO that are structurally related to SKA-31 bind in the interface between the CaM and the CAMBD to keep the channel in the open state. However, we do not wish to exclude that other allosteric effects may account for the functional antagonism observed here. Regarding selectivity, micromolar concentrations of RA-2 did not interfere with (block or activate) a series of members of the CaK1 channel families, suggesting considerable selectivity for KCa2/3 over other K+ channels.

The utility of RA-2 as a new tool and/or drug candidate for in vivo use was demonstrated by the results of the present ex vivo and in vivo experiments on freshly isolated PCAEC and coronary arteries. The patch-clamp experiments showed that endogenous KCa2 and KCa3.1 currents in PCAEC were fully inhibited by RA-2, in this regard similar to 13b. The results from myography on PCAs showed that similar to 13b (Oliván-Viguera et al., 2013) RA-2 did not modulate basal tone, suggesting that KCa2/3 channels were not essentially involved in the control of basal arterial tone at least under these ex vivo conditions. Agonist-induced EDH-type relaxations independent of nitric oxide are known to require the activation of KCa2/3 channels and subsequent smooth muscle hyperpolarization in many vascular beds (Edwards et al., 2010). Here, we found that RA-2 inhibited BK-induced EDH-type relaxation in the presence of the vasospasmic agent, U46619. This inhibition was almost complete and the small RA-2-resistant relaxation could be explained by the contribution of other endothelium-derived relaxing factors such as eicosanoids, as shown previously (Fisslthaler et al., 1999). Nonetheless, these results showed that KCa2/3 activation by BK-induced endothelial stimulation was a critically step in EDH-type relaxation in strongly precontracted PCA, as also suggested previously by others (Ge et al., 2000). However, the specific contributions of KCa2/3 to these responses were not elucidated thus far. Regarding the potency of inhibition by RA-2, it was noteworthy that RA-2 was more efficient than the more lipophilic template 13b that did not produce significant inhibition of BK-induced relaxation of PCAs in the presence of the vasospasmic U46619 in our previous study (Oliván-Viguera et al., 2013).

Our telemetry in freely moving mice showed that the animals tolerated the RA-2 injections well at a dosage of up to 100 mg/kg per day (highest dose tested). There were no signs of acute toxicity. Alterations of arterial pressure were not...
adaptation to an increase of total peripheral resistance. Interpretation of these data is that this bradycardia might be caused by endothelial KCa2/KCa3 inhibition in resistance size arteries—and baroreceptor activation to lower cardiac output and to maintain pressure constant.

In addition to expression in neurons and arteries, expression of KCa2 channels—in particular of the KCa2.2 subtype—have been reported in atrial myocytes (Tuteja et al., 2005; Li et al., 2009) and the atrioventricular node (Zhang et al., 2008). Indeed, blockers of KCa2 have been suggested to serve as antiarrhythmic drugs by prolonging repolarization times and thereby action potential duration, finally terminating atrial fibrillation (Diness et al., 2010). Thus, one alternative explanation for the lower HR might be that cardiac KCa2 inhibition by RA-2 caused prolongation of action potential duration in atrial tissues. An additional explanation could be that RA-2 altered transmission in the atrioventricular node.

Nonetheless, the precise mechanism by which RA-2 produced bradycardia remains to be identified in future studies. At present, we do not wish to exclude that by esterase-mediated hydrolysis RA-2 could be a source of diphenols with potential antioxidative properties. However, the observation that RA-2–induced bradycardia was not seen in KCa3.1-deficient mice suggested that an inhibition of KCa3.1 channels, but not of KCa2 channels, was mechanistically involved in the WT situation.

In conclusion, we identified RA-2 as a selective pan-negative-gating modulator of KCa2/3 channels with nanomolar potency and ex vivo and in vivo activity in coronary artery endothelium and in systemic cardiovascular regulation. We presumed RA-2 to be a novel pan-negative-gating modulator of KCa2/3 channels with a drug-like profile. Moreover, RA-2 can be considered a useful tool compound to study physiologic and pathophysiologic roles of KCa2/3 channels in vitro or in vivo and may be of therapeutic utility to treat hypotension and undesired hypoperemia as well as chronic inflammation and disorders characterized by abnormal cell proliferation.

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Authorship Contributions

Participated in research design: Oliván-Viguera, Wulff, Valero, Gálvez, Díaz-de-Villegas, Badorrey, Köhler.

Conducted experiments: Oliván-Viguera, Valero, Lario, Murillo, Coleman, Brown, Badorrey, Köhler.

Performed data analysis: Oliván-Viguera, Valero, Murillo, Badorrey, Köhler.

Wrote or contributed to the writing of the manuscript: Oliván-Viguera, Gálvez, Díaz-de-Villegas, Wulff, Badorrey, Köhler.

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